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SYNTHESIS AND METABOLISM OF CARBONYL-C^{14} PYRUVIC AND HYDROXYPYRUVIC ACIDS IN ALGAE

Gerhard Milhaud, Andrew A. Benson, and M. Calvin

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Gerhard Milhaud,* Andrew A. Benson,** and M. Calvin

Radiation Laboratory and Department of Chemistry
University of California, Berkeley, California

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ABSTRACT

1. Pyruvic and hydroxypyruvic acids are metabolized by Scenedesmus.
2. The products of metabolism of pyruvic-2-C^{14} and hydroxypyruvic-2-C^{14} acids are essentially identical to those of $C^{14}O_2$ fixations.
3. Lipids are rapidly formed in the light from both substrates. In the dark the major products are intermediates of the tricarboxylic acid cycle.
4. It does not appear likely that free hydroxypyruvic acid is a photosynthetic intermediate.
5. Tricarboxylic acid cycle intermediates are formed from exogenous pyruvate as fast in the light as in the dark.

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* Present address: Laboratoire des Isotopes, Institut Pasteur, Paris, France, French Cultural Relations Fellow, 1953.

** Present address: Department of Agricultural and Biological Chemistry, Pennsylvania State University, University Park, Pennsylvania.

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INTRODUCTION

The metabolism of pyruvic acid in the light and dark by Scenedesmus has been examined with two aims: To study the relationship of its metabolism to that of the intermediates of $C^{14}O_2$ fixation, and to compare its metabolic fate with that of hydroxypyruvate.

The metabolism of hydroxypyruvic acid was compared with that of $C^{14}O_2$ fixation in order to ascertain whether it might be a photosynthetic intermediate or a substrate for the enzyme systems present. Since phosphohydroxypyruvate is a possible carboxylation product of ribulose diphosphate, the metabolism of the free acid was considered of interest. Stafford et al.[1] observed high D-glyceric acid dehydrogenase activity in a variety of leaves, and suggested the possibility of its function in pentose phosphate carboxylation. The present work suggests rather that hydroxypyruvic acid may act largely as a substrate for transketolase or a hydroxypyruvic acid oxidase.[2]

Pyruvate-2-C^{14} and hydroxypyruvate--2-C^{14} were prepared on a small scale. The green alga, Scenedesmus, was allowed to photosynthesize in the presence of these substrates at low pH consistent with optimum assimilation of relatively strong organic acids. The products formed in the algae during light and dark periods were examined by two-dimensional paper chromatography.

EXPERIMENTAL

Pyruvic Acid-2-C^{14}

Carbonyl-labeled pyruvic acid was synthesized according to the method of Anker.[3] Sodium acetate was converted to acetyl bromide by heating over benzoyl bromide.[4] The acetyl bromide was distilled in vacuo and converted to acetyl cyanide upon standing at room temperature over cuprous cyanide. An ethereal solution of acetyl cyanide saturated with hydrogen chloride was hydrolyzed during thirty minutes at 0° by addition of the theo-

retical amount of water to crystalline pyruvamide. This was hydrolyzed to pyruvic acid, which was stored as the crystalline sodium salt. Its specific activity was 17.5 μc/mg; the theoretical value, based upon the barium carbonate used, was 18.2 μc/mg.

Chromatography of Pyruvic Acid-C^{14}

The labeled pyruvate gave one major spot containing 98% of the radioactivity with R_f 0.59 in phenol and R_f 0.43 in butanol-propionic acid solvent. The minor spot R_f 0.25 x 0.35 accounted for no more than one percent of the total activity.

Light Metabolism of 2-C^{14}-Pyruvate by Scenedesmus

One-day-old Scenedesmus was suspended in fresh nutrient (10% suspension) and acidified to pH 3.5 with dilute HCl. After 15 min photosynthesis in air, 1.0 mg (17.5 μc/mg) pyruvic acid per 100 mg cells was added and the air stream was continued for the duration of the fixations until the algae were rapidly filtered (3 sec) through Celite and killed with hot 80% ethanol. The solid remaining was extracted with 100% and 20% ethanol. The extracts were combined and concentrated. In a 45-minute light experiment at pH 3.5, 13% (660,000 cpm) of the pyruvate was fixed in the soluble fraction (Fig. 1), while in a 40-minute dark fixation (Fig. 2) 7% was fixed in the solubles. It would appear that CO_2 fixation in the light diminished the total fixation of pyruvic acid and diluted the labeled reservoirs of intermediates.

The concentrated extracts were chromatographed two-dimensionally and radioautographs were prepared. The products observed in the radiograms of several 35 to 45-min light-fixation experiments were the same. The lipids contained a major fraction of the radioactivity; glutamate had more than twice as much as any other single product. Other products included aspartate, malate, glutamine, succinate, phosphate esters involved in hexose synthesis, sucrose, alanine, serine, glycine, and citrate, with radioactivity decreasing in that order. No appreciable amount of glycolic acid appeared. Sucrose was hydrolyzed and the products were cochromatographed with carrier fructose and glucose. Although the 45-min light fixation included some pyruvate, its presence was apparently due to incomplete washing of the cells.

A marked difference was observed in the lipid and phospholipid areas

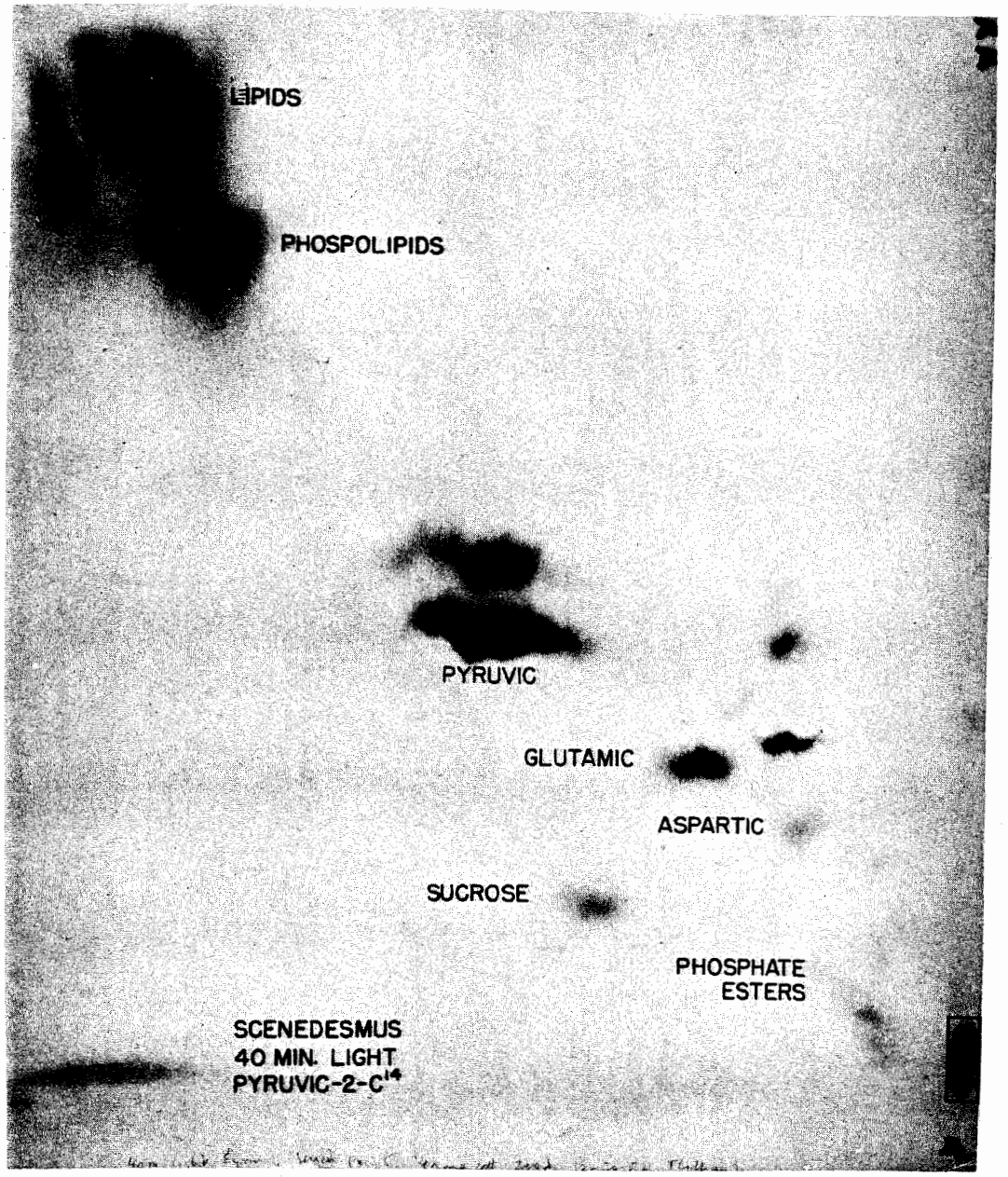

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Fig. 1 Products of light metabolism of pyruvic acid-2-C^{14}. The chromatogram was developed to the left in phenol and upwards in butanol-propionic acid.

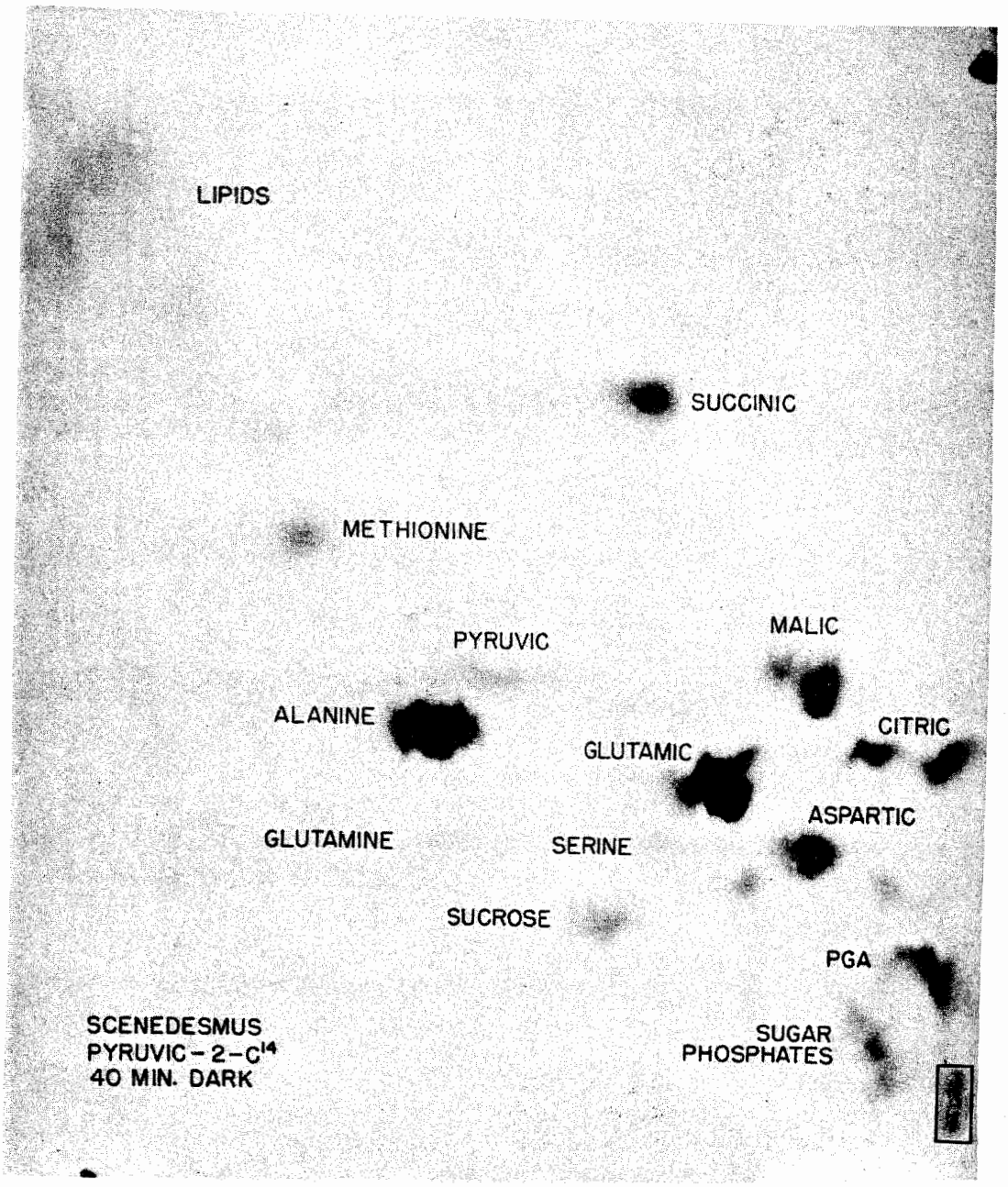

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Fig. 2 Products of dark metabolism of pyruvic acid-2-C^{14}.

from the light and dark fixations. In the light (35 min) three times as much lipid (3,200 cpm) is formed as in the dark (1,100 cpm, 40 min) although glutamic acid was labeled to the same extent in both cases. The phospholipid areas from chromatograms of both experiments had approximately equal radioactivities (1,500 cpm dark, 1,600 cpm light).

A more hydrophylic group of substances associated chromatographically with the sulfolipids (lower R_f than phospholipids) had one-fourth as much activity as the fats and was not significantly different with illumination.

It is apparent from a comparison of these radiograms with those of normal $C^{14}O_2$-labeled photosynthesis intermediates that sucrose and its phosphorylated precursors have a much lower specific activity than do glutamate and aspartate. This would infer a dilution by nonlabeled polysaccharide reservoirs. In the light, such dilution does not seriously affect incorporation of pyruvate-C^{14} into tricarboxylic acid cycle intermediates.

Dark Metabolism of Pyruvate-2-C^{14}

Dark pyruvate-C^{14} metabolism differs markedly from that of $C^{14}O_2$. The products (Fig. 2) include many of those characteristic of $C^{14}O_2$ photosynthesis, in addition to those respiration intermediates which become labeled by exchange in dark $C^{14}O_2$ fixations.[5]

The products seen in the radiograms included sucrose and hexose phosphates in quantities exceeding those formed with $C^{14}O_2$ in the dark. Sucrose was identified by hydrolysis and chromatography. Monophosphates of galactose, mannose, glucose, and fructose were identified by phosphatase hydrolysis and cochromatography of the free hexoses. The products differed strikingly from those of photosynthesis in that neither sedoheptulose monophosphate nor ribulose diphosphate was observed. Uridine diphosphate glucose was observed and contained the major amounts of labile glucose phosphate. Lipids were observed but not in the considerable amounts formed in the light.

Bromopyruvic Acid

The preparation described by Sprinson and Chargaff[6] was adapted to small-scale reactions. Pyruvic acid is converted to the corresponding bromopyruvic acid, and the bromopyruvic acid is hydrolyzed just before use to the hydroxypyruvic acid. To 130 mg of pyruvic acid at 50°C, 244 mg of liquid bromine was added. As the reaction starts, the bromine disappears and hydrobromic acid is formed. The mixture was placed in a

vacuum desiccator over moist sodium hydroxide pellets and the solvent was removed by suction. Bromopyruvic acid crystallized and 190 mg was recovered after drying (equivalent to 77%). The bromopyruvic acid had a specific activity of 10.5 ±1μc/mg compared to the calculated 11.5 μc/mg. The dilution may be due to the formation of some dibromopyruvic acid.

Paper Chromatography of Bromopyruvic Acid

Crystalline bromopyruvic acid gave a single spot when run in phenol (R_f 0.49) and butanol-propionic acid (R_f 0.65) on Whatman No. 4 paper. When the preparation was made alkaline, hydroxypyruvate appeared at once. In the presence of sodium ion, bromopyruvate solutions are observed to form double spots upon phenol development on Whatman No. 1 paper.

Chromatographic Properties of Hydroxypyruvic Acid*

Bromopyruvic acid was hydrolyzed in dilute alkali as done by Sprinson, or in phosphate buffer when small quantities of radioactive material were involved. Three major labeled products resulted when bromopyruvic acid was hydrolyzed in mild alkali. When the bromo acid was hydrolyzed with 0.1 N sodium hydroxide by slow addition to maintain pH 7 to 8, the radiogram had four spots, with R_f values in phenol and butanol-propionic acid for A, 0.15 x 0.35; B, 0.31 x 0.38; C, 0.31 x 0.64; and D, 0.44 x 0.64. A and B (hydroxypyruvate) contained over 80% of the C^{14}. From the slight streaking observed in the second (butanol-propionic) direction and the identity of C (by cochromatography) with bromopyruvate, it may be concluded that residual bromopyruvate had been hydrolyzed upon drying the phenol or upon acidic action by propionic acid to give a good yield of hydroxypyruvate.

A hydrolysis in pH 6.0 phosphate buffer gave hydroxypyruvate and glycolate with very little residual bromopyruvate. The phenol R_f values, of course, were changed somewhat with the pH. Spot D has been identified as glycolic acid. This apparently arises from decarboxylation of tartronic semialdehyde followed by air oxidation.

Both bromopyruvate and hydroxypyruvate give single spots after treatment of the solution with Dowex-50 cation resin to remove sodium ion. No

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* We are indebted to Mr. R. A. Sherrer for an examination of the chromatographic properties of hydroxypruvic acid.

evidence for separation of tautomers (dihydroxyacrylic acid and tartronic acid semialdehyde) during chromatography was observed. The major radioactive components are shown in Fig. 3.

Photosynthesis with Hydroxypyruvic Acid-2-C^{14} by Scenedesmus

Previous experience with glycolic and formic acid feeding experiments in this laboratory suggested that a low pH would favor assimilation of the labeled substrate. A three-hour and a 40-minute photosynthesis with dark-fixation controls were performed with Scenedesmus at pH 3.5. The radioactivity in the soluble products was one-third to one-fourth of that fixed in corresponding experiments with pyruvate-2-C^{14}. The presence of residual bromopyruvate in the substrate suggested a possible inhibitory effect analogous to that of iodoacetate. Hence, $C^{14}O_2$ fixations in dilute phosphoric acid (pH 3) were performed with or without hydroxypyruvate prepared by identical hydrolysis conditions from bromopyruvate. No significant effect upon products of 1 min $C^{14}O_2$ fixation by the possible bromopyruvate contaminant was observed.

The hydroxypyruvate-2-C^{14} assimilations in the light in the presence of CO_2-free air gave low yields of labeled products. These included glutamate, aspartate, lipids, sucrose. In a 60-min light experiment, however, an unusual amount of glycolic acid was observed in addition to abnormally high glycine concentrations and lesser amounts of serine, alanine, glutamic acid, and phosphate esters (Fig. 4). No glyceric acid was observed.

Degradation of Glycolic Acid from Products of 40-Minute Light Assimilation of Hydroxypyruvate-2-C^{14} in CO_2-free Air

Glycolate eluted from fresh chromatograms was degraded* according to the method of Schou et al.[7] The carboxyl carbon was found to have 89% and the α-carbon 18% of the total C^{14} in the starting acid.

DISCUSSION

The copious yield (~85%) of carboxyl-labeled glycolate from hydroxypyruvate-2-C^{14} is in accord with decarboxylation by transketolase or hydroxy-pyruvic acid oxidase,[8,2] followed by oxidation of the glycolyl derivative to free glycolic acid,

$$HOCH_2C^*OCOOH \xrightarrow{-CO_2} \xrightarrow{-2[H]} HOCH_2C^*OOH,$$

2 We are indebted to the kindness of Dr. E. M. Thain for the chemical degradation.

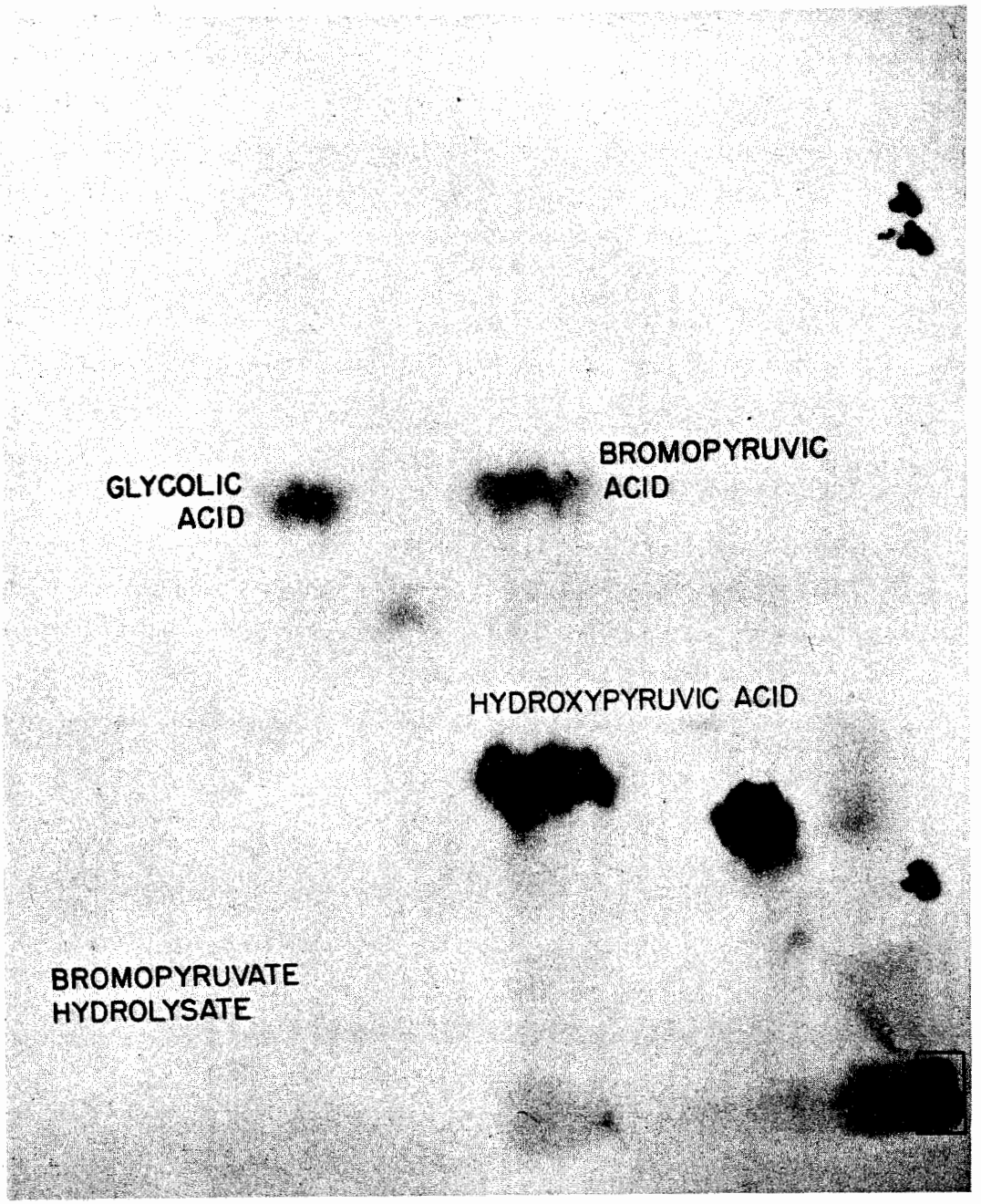

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Fig. 3 Hydrolysis products of bromopyruvic acid-2-C^{14}.

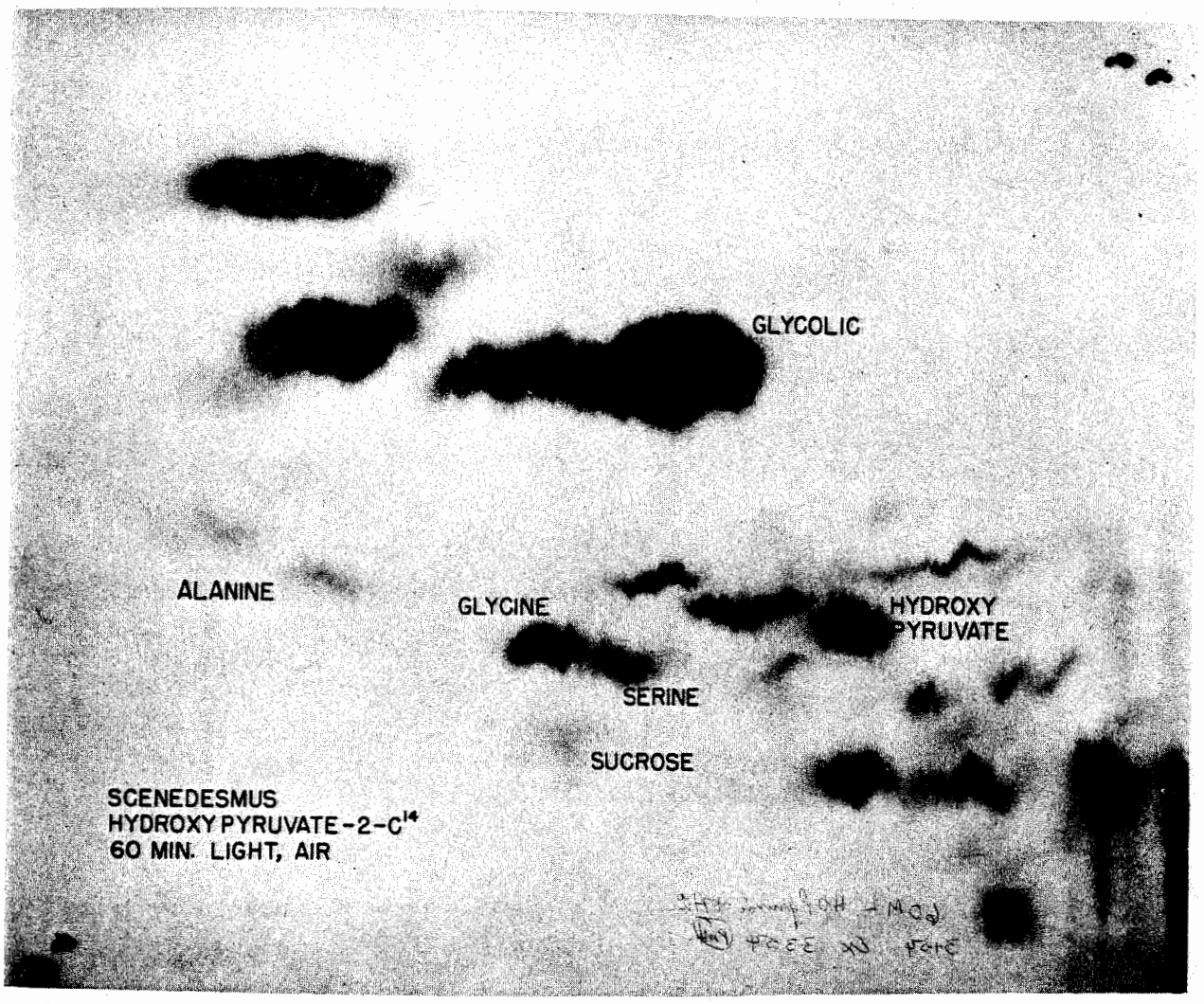

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Fig. 4 Products of light metabolism of hydroxypyruvic acid-2-C^{14}.

while the minor yield of α-labeled glycolate (~ 15%) can be accounted for by the nonenzymatic decarboxylation which is observed with preparations of hydroxypyruvate,

$$HOCH_2C^*OCOOH \rightleftharpoons OCHC^*HOHCOOH \longrightarrow CO_2 + OCHC^*H_2OH$$
$$\xrightarrow{ox.} HOOCC^*H_2OH.$$

Any reassimilation of $C^{14}O_2$ from the oxidized substrate would have resulted in symmetrically labeled glycolate.[7] Glycolic acid is readily oxidized further in Scenedesmus and hence can serve as a respiratory intermediate.

The rather slow observed formation of carbohydrates from hydroxypyruvate suggested that its aerobic metabolism by algae is largely oxidative even though enzyme systems for its incorporation are present. Pyruvate-2-C^{14} is converted to lipids and carbohydrates at least ten times as fast as is hydroxypyruvate under comparable conditions. It seems unlikely, then, that hydroxypyruvate is a normal intermediate or a source of glycolyl groups involved in carbohydrate synthesis.

The primary effect of light on pyruvate-2-C^{14} metabolism is on the rate of lipid synthesis. The rate of tricarboxylic-acid-cycle oxidation of pyruvate-2-C^{14} is not completely inhibited in the light. This suggests an independent tricarboxylic-acid-cycle metabolism for exogenous pyruvate which proceeds at a similar rate in dark and light while lipid formation is light-dependent. This appears contrary to previous observations of light inhibition of respiration of photosynthetic intermediates,[9,10] which was attributed to maintenance of thioctic acid in the reduced form incapable of pyruvate oxidation. However, since a fraction of the thioctic acid of the cell is not associated with the chloroplasts , it is likely that a separation of oxidative sites allows extra-chloroplastic pyruvate oxidation to proceed in the light as well as in the dark. The equality of light and dark respiration observed by Brown[11] for organisms with highly organized chloroplasts apparently demands such an interpretation.

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